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Research Article

STUDY OF POTENTIAL IN VITRO ANTIDIABETIC ACTIVITIES OF METHANOLIC EXTRACTS OF *Memordica charantia, Nyctanthes arbor,* and *Tinospora cordifolia* FROM DANG NEPAL

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ABSTRACT

A wide diversity of medicinal plants rich in phytochemicals are the potential source of drugs. The phytochemical composition of plants varies with the geographical location. These selected medicinal plants containing phytochemicals inhibit the catalytic action of *a*-amylase to ameliorate the complications of diabetes. The main goal of this work is to compare the phytochemical composition and biological activities such as antioxidant, toxicity, and *a*-amylase inhibitory activity of *Memordica charantia*, *Nyctanthes arbor,* and *Tinospora cordifolia*. The study employed qualitative analysis through phytochemical screening, followed by quantitative measurement of total phenolic and flavonoid content by using the Folin-Ciocalteu reagent and colorimetric method. Additionally, biological activities were assessed using the 2,2 diphenyl-1-picrylhydrazyl antioxidant assay, *a*-amylase starch iodine method, and brine shrimp lethal test. The study revealed that the total phenolic contents in *T. cordifolia*, *M. charantia*, and *N. arbor* were measured as 140.49, 101.69, and 98.76 mg GAE/g, respectively. Consequently, *N. arbor, T. cordifolia,* and *M. charantia* showed strong antioxidant properties, with IC_{50} values of 48.16, 45.07, and 69.34 µg/mL, respectively9. Furthermore, in the brine shrimp lethal test, all the extracts exhibited low toxicity, with the LC_{50} value of *M. charantia* (316.22 µg/mL), *N. arbor* (275.42 µg/mL), and *T. cordifolia* (275.42 µg/mL), respectively. GC-MS analysis of hexane fraction of *N. arbor* methanol extract reveals the presence of compounds like Hexanoic acid, 6-amino-6-oxo-, and Benzo[b]thiophene-2-carboxamide,3-chloro-N-(2-bromophenyl). Therefore, in all the aspects of the study, the methanolic extract of *M. charantia*, *N. arbor* and *T. cordifolia* were potent, which concludes that these extracts contain abundant phytochemicals and possess diverse biological activities.

Keywords: Antioxidant assay, GC-MS, *M. charantia*, *N. arbor*, phytochemicals, *T. cordifolia*, *a*-amylase

INTRODUCTION

Medicinal plants offer a valuable source of phytochemicals that possess pharmacological and biological activities, making them suitable candidates for drug development. Ayurvedic medicine possesses various tonic qualities like antibacterial properties, antidiabetic activity, and anemia treatment which is a mixture of various medicinal plants. Traditionally, *Memordica charantia* (commonly known as bitter gourd) has been employed in Asia, South America, India, the Caribbean, and East Africa for the treatment of diabetes (Wang *et al.*, 2019). This plant has demonstrated various therapeutic properties, including hypoglycemic, antidiabetic, antioxidant, antiviral, antimalarial, and antimicrobial activities (Chokki *et al.,* 2020). *Nyctanthes arbor*, commonly known as night-flowering jasmine, has a rich history in Ayurveda where its leaves have been utilized for managing chronic fever, obstinate sciatica, cough, malaria, constipation, intestinal worm infestations, and excessive diuresis (Saxena *et al.*, 1987). *Tinospora cordifolia*, another medicinal plant, has been extensively used in folk and Ayurvedic medicine (Choudhry *et al.*, 2014). Its medicinal properties include the reduction of glucose levels, relief from visceral obstruction (Kapil & Sharma, 1997), anti-inflammatory effects, antioxidant activity, antiallergic properties, anticancer potential, antipyretic effects, hepatoprotective activity, immunomodulatory properties, diuretic effects, and protection against the toxicity of cancer in chemotherapy (Panchabhai *et al.*, 2008).

Moreover, many diseases, including diabetes, cancer, and high blood pressure, are initially started by oxidative stress (Sen & Packer, 1996), which results from a normal metabolic process triggered by various environmental factors. This process generates highly reactive molecules known as reactive singlet oxygen species, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), hydroxyl radical ($^{\bullet}OH$) and superoxide anion (O^{2-}), which can damage living cells Forman, (2016), Sies, (1986). Diabetes, characterized by defective insulin secretion, insulin resistance, or a combination of both, is a prevalent and rapidly growing global health concern (Ahmed *et al.*, 2016). It is estimated that by 2045, approximately 629 million individuals between the ages of 20 and 79 will be affected by diabetes (Cho *et al.,* 2018). Interestingly, synthetic drugs like acarbose available for diabetes treatment often exhibit undesirable side effects. There is a need for alternative medicines with fewer or no adverse effects.

Based on these medicinal properties of plants and their potential for managing various health conditions, there remains a significant gap in understanding the regional variation in phytochemical composition and bioactivities of medicinal plants, particularly those found in less studied geographic regions like western Nepal. And the unique environmental factors such as altitude, soil type, and climatic conditions of western Nepal may influence the phytochemical profile and therapeutic efficacy of its

medicinal plants. Addressing this gap could provide valuable insights into the role of geography in enhancing or modulating the medicinal properties of these plants, offering alternative treatments with minimal side effects for diseases like diabetes and other oxidative stress-related conditions. In this study, we selected three plants to conduct phytochemical analysis such as total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity, and biological activities such as *a*-amylase inhibition, brine shrimp bioassay. Additionally, we employed techniques such as column chromatography, Fourier-transform infrared spectroscopy (FTIR), and gas chromatography-mass spectrometry (GC-MS) to isolate and identify compounds during our analysis.

MATERIALS AND METHODS

Chemicals and Equipment

Solvents such as ethanol, methanol, hexane, Folin-Ciocalteu reagent (FCR), dimethyl sulphoxide (DMSO), and iodine of analytical grade were purchased from Scientific Fisher Company. Other chemicals such as potassium hydroxide (KOH), Sodium hydroxide (NaOH), aluminum tri-chloride ($AlCl_3$), disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), and sodium dihydrogen phosphate (NaH_2PO_4) were bought from Merck Company, India. Similarly, Porcine pancreatic *a*-amylase enzyme, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, and quercetin was purchased from Sigma-Aldrich, USA. Acarbose is a standard drug imported from Canvax Biotech Company, Germany. Digital weighing balance (GT210), hot air oven (griffin-Grundy), rotatory evaporator (Buchi RE111) with water bath (Buchi 461), Spectrophotometer (WpA, supplied by Philip Harris Shenstone, England), GCMS-QP2010 Ultra SHIMADZU, cuvettes, beakers, conical flasks, test tubes, reagent bottles, were also used in this work. The experiments were conducted at the Organic Chemistry laboratory, Central Department of Chemistry, Tribhuvan University, Kirtipur, Nepal, 2018.

Collection and Identification of Plant Samples

The fresh leaves of *M. charantia*, *N. arbor*, and *T. cordifolia* were collected from Lamahi, located in the Dang district, Nepal, March 2018, which is clearly shown in the map of Nepal in Figure S3b. The taxonomic identification of these plants took place at Tribhuvan University's Central Department of Botany in Kirtipur, Nepal. The freshly collected leaves were carefully cleaned with water, dried in shade, finely grounded by using an electric grinder, and stored in clean plastic bags. Detailed descriptions of collected plants are given in Table 1.

Table 1. Description of selected Medicinal plant

Scientific name	Family	Part used	Traditional Usage	References
Memordica charantia	Cucurbitaceae	Leaf	Use for hypoglycemic, antidiabetic, antiviral, antimalarial, and antimicrobial activities	(Wang *et al.*, 2019)
Nyctanthes arbor	Oleaceae	Leaf	Use for chronic fever, obstinate sciatica, cough, malaria, constipation, intestinal worm infestations, and excessive diuresis	(Saxena *et al.*, 1987)
Tinospora cordifolia	Menispermaceae	Leaf	Use to reduce glucose levels, visceral obstruction, anti-inflammatory, antiallergic, antipyretic, hepatoprotective, diuretic, and protection to toxicity of cancer in chemotherapy	(Choudhry *et al.*, 2014; Kapil & Sharma, 1997)

Note: - These medicinal plants were collected by ethnomedicinal approach from Lamahi, Dang district, Nepal

Cold percolation

Phytochemical extraction was performed using the cold percolation method with methanol. A total of 100 g of powdered leaves from each plant was mixed with 350 mL of methanol in conical flasks and kept in the dark for 24 hours. The mixtures were then filtered 5–7 times using Whatman filter paper, and the filtrates were concentrated under reduced pressure at 55 ºC using a rotary evaporator. The crude extracts were dried in Petri dishes, and the percentage yield was calculated using the formula.

$$\% \text{ Yield of extract} = \frac{\text{Wt.of extract obtained } (g)}{\text{Wt. of the powder taken} (g)} \times 100\% \quad \ldots\ldots\ldots\ldots (1)$$

Phytochemical Analysis

The presence of volatile oil, alkaloids, terpenoids, coumarins, flavonoids, quinones, polyphenols,

glycosides, reducing sugars, saponins, and tannins in the methanolic extracts of the plants was determined according to the protocol (Das *et al.*, 2018).

Total Phenolic Content

The analysis of total phenolic content (TPC) was conducted by following a standardized protocol (Ainsworth & Gillespie, 2007). Initially, a stock solution of the extract was prepared by dissolving 0.01 g extract in 10 mL of methanol. From this stock solution, various concentrations (20, 40, 60, 80, 120, and 160 µg/mL) were obtained through serial dilution with methanol. Similarly, a stock solution of gallic acid (1000 µg/mL) was prepared by dissolving 10 mg gallic acid in 10 mL of methanol. The different concentrations (20, 40, 60, 80,120, and 160 µg/mL) of gallic acid were prepared by diluting the stock solution. Then, 1 mL of the gallic acid solution from each concentration was pipetted out separately to test tubes. To each of these test tubes, 5 mL of 10% Folin-Ciocalteu reagent (FCR) and 4 mL of 7% sodium carbonate solution were added, resulting in a final volume of 10 mL. The resulting blue color mixture was thoroughly shaken and incubated for 30 minutes in a water bath. Subsequently, the absorbances of the solutions were measured at 760 nm using a UV-visible spectrometer against a blank solution containing all reagents except for gallic acid. All experiments were performed in triplicate. Following the same procedure, the absorbance of the extracts was measured, and the results were recorded.

The total phenolic content was calculated using the following relation:

$$C = \frac{cV}{m} \quad \text{.....(2)}$$

Here,
C = total content of the phenolic compounds (mg/g) in gallic acid equivalent (GAE)
c = concentration of gallic acid established from the calibration curve (mg/mL)
V = volume of extract (mL)
m = mass of extract (initial)

Total Flavonoid Content

The determination of total flavonoid content (TFC) in the plant extracts was conducted by using the aluminum chloride colorimetric method, with slight modifications on the standard protocol from previous paper (Chemjong & Subba, 2022). Quercetin was taken as the positive control for reference. Initially, a stock solution of the extract was prepared by dissolving 0.01 g of the extract in 10 mL of methanol. From this stock solution, various concentrations of the extract (20, 40, 60, 80, 120, and 160 µg/mL) were obtained through serial dilution. Similarly, a stock solution of quercetin was prepared by dissolving 20 mg of quercetin in 2 mL of methanol. Serial dilutions were performed to obtain different concentrations of quercetin (20, 40, 60, 80, 120, and 160 µg/mL) from the stock solution. Furthermore, 1 mL of each concentration of quercetin solution was pipetted out into separate test tubes, each containing 4 mL of distilled water. At the start of the reaction (zero time), 0.3 mL of 5% sodium nitrite solution was added to each tube. Then, after 5 minutes, 0.3 mL of 10% aluminum trichloride solution was added, and after 6 minutes, 2 mL of 1 M sodium hydroxide solution was loaded. The final volume of the mixture was adjusted to 10 mL by adding 2.4 mL of distilled water. Finally, the absorbance of the resulting, pink-colored mixture was measured at 510 nm against a blank solution containing all reagents except quercetin. The absorbance of the extract was measured using the same procedure as for quercetin.

The total flavonoid content was calculated using the following relation:

$$C = \frac{cV}{m} \quad \text{.....(3)}$$

Here, C = total flavonoid content (mg/g) in quercetin equivalent (QE); c = concentration of quercetin established from the calibration curve (mg/mL); V = volume of extract (mL)

Antioxidant Assay

The antioxidant activity of the plant extracts was analyzed using the DPPH free radical scavenging assay, by following the method from (Subedi *et al.*, 2012). Initially, 0.0078864 g of 2,2-diphenyl-1-picrylhydrazyl (DPPH) crystal was dissolved in distilled water in a 100 mL volumetric flask to prepare a 0.2 mM DPPH solution, which was then stored in the dark for future use. A stock solution of ascorbic acid at a concentration of 1000 µg/mL was prepared by dissolving 10 mg of ascorbic acid in 10 mL of methanol. From the stock solution of ascorbic acid, various concentrations (5, 10, 20, 40, 80, and 100 µg/mL) were prepared. Similarly, stock solutions of the plant extracts (1000 µg/mL) were prepared by dissolving 10 mg of the extract in 10 mL of methanol. These stock solutions were further diluted to obtain different concentrations (5, 10, 20, 40, 60, 80, and 100 µg/mL). Subsequently, 2 mL of the ascorbic acid solution was pipetted out and mixed with 2 mL of the 0.2 mM DPPH solution. The mixture was then kept in the dark for 30 minutes. For the blank, 2 mL of methanol was mixed with 2 mL of the 0.2 mM DPPH solution and treated in the same manner. Then, the absorbance of the ascorbic acid solutions was measured at 517 nm using a spectrophotometer. Similarly, the absorbance values of the plant extracts were measured following the same procedure as described above. Finally, the level of scavenging activity against free radicals was assessed using Equation (4). Additionally, a standard graph of ascorbic acid was created and compared to the extract, considering their respective IC_{50} values.

$$\%\ \text{Free radical scavanging activity} = \frac{A^0 - A}{A^0} \times 100\% \quad \text{.....(4)}$$

Here, A^o = absorbance of the blank; A = absorbance of the sample

Brine Shrimp Bioassay

The evaluation of toxicity of compounds present in the selected plants was conducted by using *Artemia salina* eggs with a procedure (Meyer *et al.,* 1982). This method is a simple bioassay for natural product research, which determines the LC_{50} values (µg/mL) for the methanolic extracts. Compounds exhibiting LC_{50} values below 1000 ppm (µg/mL) are considered pharmacologically active. To initiate the process, artificial seawater was prepared by dissolving sodium chloride (23.50 g/L), Na_2SO_4 (4 g/L), KCl (0.68 g/L), H_3BO_3 (0.27 g/L), $MgCl_2.2H_2O$ (10.68 g/L), $CaCl_2.2H_2O$ (1.48 g/L), $NaHCO_3$ (0.197 g/L) and Na_2EDTA (0.0003 g/L), in one liter of distilled water. A beaker containing the artificial seawater was sprinkled with 50 mg of brine shrimp eggs and covered with aluminum foil featuring small pores to allow for heat and light. The beaker was then set aside at room temperature, illuminated with a 40W bulb, and left for 48 hours to allow the eggs to hatch into larvae.

Following hatching, 20 mg of the extract in 2 mL of methanol was dissolved to prepare a stock solution, which was subsequently diluted to concentrations of 1000, 100, and 10 µg/mL. Additionally, 2 mL of solution from each concentration was transferred to nine test tubes, with three test tubes allocated to each concentration. Similarly, three test tubes containing 2 mL of methanol were prepared as blanks. Subsequently, all the test tubes were allowed to undergo solvent evaporation for 24 hours. Once the solvent had completely evaporated, 5 mL of artificial seawater was added to each test tube, and the solution was thoroughly mixed to suspend any residue. Then, 10 mature brine shrimp nauplii were introduced into all the test tubes. After 24 hours, the number of surviving organisms was counted using disposable pipettes. Finally, the LC_{50} value (the lethal concentration dose required to kill 50% of the organisms used in the bioassay) was calculated as follows (Aryal & Shakya, 2023).

If n is the number of replicates (n=3), x is the log of the concentration of the solution in µg/mL (log 10, log 100 and log 1000) and y is the probit for average survivors for all replicates, then we have:

$$\alpha = \frac{\sum y - \sum x}{n} \quad \text{..... (5)}$$

$$\beta = \frac{\sum_{xy} - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} \quad \text{..... (6)}$$

Now, from Probit regression.

$$Y = \alpha + \beta X \quad \text{..... (7)}$$

$$X = \frac{Y - \alpha}{\beta} \quad \text{.....(8)}$$

Here, Y is a constant having value 5 for calculating LC_{50} value.

Hence,

$$LC_{50} = \text{antilog } (X) \quad \text{..... (9)}$$

α-amylase Inhibition

a-amylase inhibition activity of selected plant extracts was evaluated by using the starch iodine method with slight modifications to the standard protocol (Xiao *et al.*, 2006). In this assay, *a*-amylase served as the enzyme, starch as the substrate, and acarbose as the positive control. To begin, a 0.02 M phosphate buffer was prepared using disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), and 6.7 mM sodium chloride. Likewise, a 0.0025 M iodine solution was prepared by dissolving 0.064 g of iodine in a 2% KI solution, and a 0.1 M HCl solution was prepared in a 100 mL volumetric flask. Then, the stock solution of extract and acarbose were prepared separately by dissolving 10 mg of the plant extract and acarbose in 10 mL of dimethyl sulphoxide (DMSO). Both the stock solutions were further diluted to concentrations of 1000, 640, 320, 160, 80, and 40 µg/mL respectively. Additionally, a 5% starch substrate was prepared in the phosphate buffer. Solution of *a*-amylase with a concentration of 50 µg/mL was prepared by dissolving 5 mg of 3U/mL *a*-amylase in 100 mL of the phosphate buffer. For the analysis, 400 µL of phosphate buffer, 400 µL of the starch solution was incubated along with 200 µL of various concentrations (1000, 640, 320, 160, 80, and 40 µg/mL) of the plant extract and acarbose separately at 37 ºC for 5 minutes in a test tubes. Subsequently, 200 µL of the *a*-amylase solution was added to each incubated mixture, and the contents were further incubated for 15 minutes at 37 ºC. After the incubation period, the enzymatic reaction was quenched by adding 800 µL of 0.1 M HCl. Finally, 1000 µL of the iodine reagent was added to maintain the final volume of 3 mL, and immediately the absorbance was measured at 630 nm. The absorbance was taken in a triplicate manner, and the percentage inhibition of the enzyme was calculated by using the Equation (10).

$$\% \text{ Inhibition} = \frac{Abs_2 - Abs_1}{Abs_4 - Abs_3} \times 100\% \quad \text{.....(10)}$$

Here, Abs_1= absorbance of an incubated mixture containing plant extract, starch, and amylase; Abs_2= absorbance of an incubated mixture containing plant extract and starch; Abs_3= absorbance of an incubated mixture containing starch and amylase; Abs_4= absorbance of an incubated solution containing starch only

Column Chromatography

100 g of silica gel was soaked in hexane overnight. The soaked silica gel was then packed into a column measuring 3.3 mm in internal diameter and 40 cm in length to prepare the stationary phase. To prepare a concentrated extract solution, 8 g of previously dried methanol extract of *N. arbor* was dissolved in hexane to get hexane fraction. The resulting concentrated extract (hexane) was carefully introduced at the top of the

packed column, allowing it to adsorb onto the stationary phase undisturbed. The column was then initially eluted with hexane, followed by a gradient of hexane in ethyl acetate with increasing polarity. Finally, the elution proceeded up to 100% ethyl acetate, after which the polarity was further increased to 50% methanol in ethyl acetate. The fractions eluted from the column were collected in labeled individual beakers for subsequent analysis using thin-layer chromatography (TLC). The details of the eluent polarity, corresponding fractions, and TLC reports were also analyzed, which is reported in Table 6.

Identification and Characterization of Compounds

Upon analyzing the elution fractions 11-15 using TLC, multiple spots were observed. However, when using 13% ethyl acetate in hexane as the eluent, a single spot was observed for fractions 31-35. Consequently, fractions 31-35 were evaporated to yield a solid yellow crystalline compound, which was subsequently washed with hexane multiple times. To identify the functional groups in the compound, FTIR analysis was conducted. Additionally, the hexane fraction of *N. arbor* leaves underwent GC-MS analysis at the National Forensic Science Laboratory (NAFOL) in Khumaltar, Lalitpur, to identify the major compounds. The shimadzu single quadrupole GCMS-QP2010 plus: Gas chromatograph-Mass Spectrometer (GC-MS) was used. Helium was used as carrier gas. All chromatographic separations were performed on a capillary column (Rtx-5MS Low- Bleed GC Capillary Column), having specifications: length; 30 m, thickness; 0.25 mm, ID; 0.25µm. Other GC-MS conditions are: ion source temperature (EI); 200 °C**,** interface temperature; 280 °C, pressure; 76.9 kPa, solvent cut time; 2 min. 1 µl of sample was injected into the GC column. The injector was operated in a split mode in the split ratio 10.0 Injection temperature was 250°C. The column temperature program started at 110°C for 2 min and changed to 200 °C. The temperature was raised to 280 °C and held for 9 min. Total elution time 36 min.

Statistical Analysis

During the analysis, all calculations were evaluated by using GraphPad Prism 8.0.2.

RESULTS AND DISCUSSION

Phytochemical Analysis and percentage yield

Table 2**.** Presents the outcomes of the qualitative phytochemical screening conducted on the methanolic extract of the selected plants. It clearly shows that flavonoid, polyphenol, and Quinone are present in all the selected medicinal plants. The supplementary material provides a comprehensive explanation of the procedures followed for these tests. Thus, the percentage yield of the methanol extracts from the leaves of *M. charantia*, *N. arbor*, and *T. cordifolia* were found to be 8%, 12%, and 10% (w/w), respectively, which was illustrated in Table 3.

Table 2. Comparative study of phytochemicals present in Methanol extracts of the plants with literature findings

Class of Phytochemicals	*M. charantia*			*N. arbor*		*T. cordifolia*		
	Present study	Abdillh *et al.*(2015)	Das *et al.*(2014)	Present study	Paudel *et al.*(2014)	Present study	Jamdar *et al.*(2014)	Kaur *et al.*(2016)
Volatile oil	-	-	-	-	-	-	-	-
Alkaloids	-	-	-	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+
Coumarins	-	-	-	-	-	-	-	-
Flavonoids	+	+	+	+	+	+	+	+
Quinones	+	+	+	+	+	+	+	+
Polyphenols	+	+	+	+	+	+	+	+
Glycosides	-	-	-	+	+	+	+	+
Reducing sugars	-	-	-	-	-	-	-	-
Saponins	+	+	+	+	+	+	+	+
Tannins	+	+	-	+	+	+	+	+

Note: - Comparative analysis includes the comparisons of current finding phytochemical constituents with the previous studies, + sign denotes present and – sign denotes absent of phytochemical

Table 3. Physical features, percentage yield, TPC and TFC of plants in Methanol extracts

Name of plant	Color of crude extract	Percentage yield (%)	TPC (mg GAE/g)	TFC (mg QE/g)
M. charantia	Green	8%	101.69 ± 4.84	15.40 ± 1.654
N. arbor	Dark brown	12 %	98.76 ± 3.36	37.94 ± 8.15
T. cordifolia	Dark Green	10 %	140.49 ± 4.57	48.13 ± 4.56

Note: - Quantitative analysis of phytochemicals in the selected medicinal plants such as Percentage yield, TPC, and TFC.

Phytochemical screening of plant extracts unveiled a variety of compounds. In the case of *M. charantia*, terpenoids, flavonoids, quinones, polyphenols, saponins, and tannins were detected, consistent with the findings (Abdillah *et al.*, 2015). However, Das *et al.* (2014) reported the absence of tannins, indicating a variation in phytochemical profiles influenced by environmental factors such as air, water, soil, minerals, and the

extraction process, as suggested by (Sarkar & Ghosh, 2017). *N. arbor* and *T. cordifolia* shared similar phytochemicals with *M. charantia* along with alkaloids and glycosides, while volatile oils and sugars were absent in all three plants. Our findings for *T. cordifolia* align with the studies conducted by (Jamdar *et al.*, 2014) and Kaur *et al.*, (2016). Similarly, our results for *N. arbor* were consistent with the phytochemical constituents identified by (Paudel & Gyawali, 2014).

Total Phenolic Content

The estimation of the total phenolic content in the methanolic extract was performed by using the Folin-Ciocalteu reagent, with gallic acid as a positive control. The linear regression equation, $y = 0.01440x + 0.001467$ ($R^2 = 0.9951$), derived from the gallic acid standard curve (Figure 1a), was utilized to determine the concentration of gallic acid (See Table S3a). Equation (2) was used to calculate the total phenolic content (TPC) in the extract (See Table S3b). The TPC was expressed in milligrams of gallic acid equivalents (GAE) per gram of dry weight. Table 3 displays the calculated TPC values for the plant extracts. Among the extracts, *T. cordifolia* exhibited the highest concentration of phenolic content (140.49 ± 4.57 mg GAE/g). Comparatively, *M. charantia* had a slightly higher concentration (101.69 ± 4.84 mg GAE/g) than *N. arbor* (98.76 ± 3.36 mg GAE/g), as depicted in Figure (1b) in the form of a bar diagram. Thus, the order of total phenolic content in the selected plant extracts were *T. cordifolia* > *M. charantia* > *N. arbor*.

The results of our current study were compared to previous research findings. According to Rathee *et al.,* the methanol extract of *N. arbor* had a total phenolic content of 231 ± 0.81 GAE per gram (Rathee *et al.,* 2007), which was higher in concentration compared to our obtained result. Similarly, Khan *et al.* reported a total phenolic content of 195.45 ± 18.88 mg GAE/g in the methanol extract of *T. cordifolia* (Khan *et al.*, 2020), which was significantly higher than our result shown in Figure (1b). Another study by Chokki *et al.* (2020) found a total phenolic content of 989.55 ± 28.16 µg GAE/mg in the methanol extract of *M. charantia*, slightly higher than our results. Interestingly, Prasad and Chauhan (2019) discovered that the methanolic extract of *T. cordifolia* root and stem contained 0.2655 and 0.2639 mg/g, respectively, which is significantly lower than the results obtained in this study. These variations in phenolic content could be attributed to environmental factors such as soil type, temperature, and rainfall (Kumar, 2016). Additionally, differences in the extraction process, duration, and experimental setup (Sarkar & Ghosh, 2017) may have contributed to the varying values of phenolic content in the selected plant extracts.

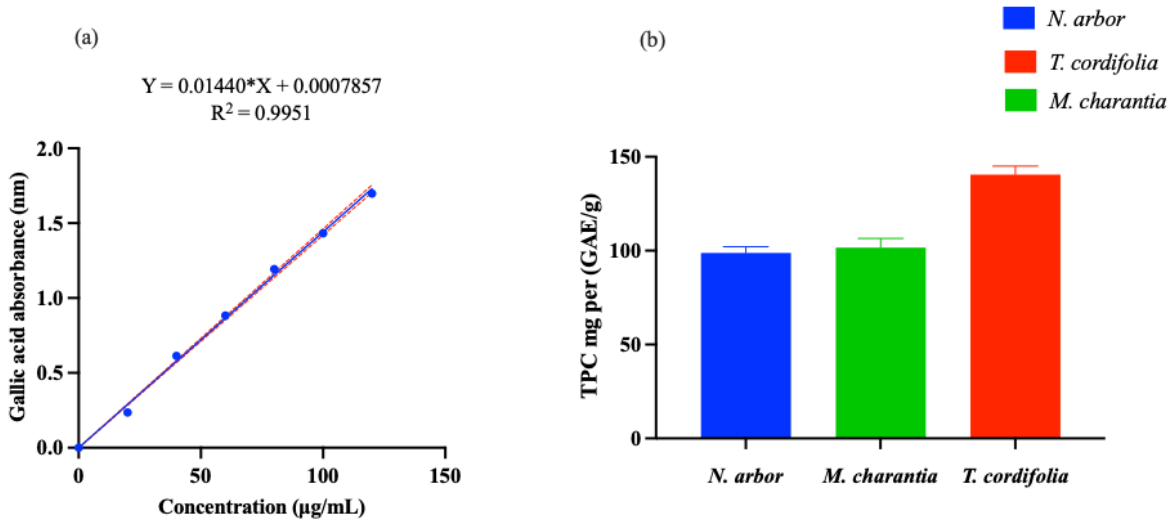

Figure 1. (a) Gallic acid standard curve; (b) Bar-graph showing the total phenolic content (mg GAE / gram of dry extract).

Total Flavonoid Content

The quantification of total flavonoid content in a plant extract was calculated by constructing a standard curve of quercetin, as illustrated in Figure (2a) (See Table S3c). The linear regression equation $y = 0.001514x - 0.002964$, $R^2 = 0.9904$, derived from the standard graph gives an unknown concentration of extract, which was employed to evaluate the total flavonoid content in the selected plant extracts using Equation (3) (See Table S3d). The results obtained were expressed as mg of quercetin equivalent (QE) per gram of extract in dry weight. Accordingly, the flavonoid content in *M. charantia*, *N. arbor*, and *T. cordifolia* were found to be 15.40 ± 1.65, 37.94 ± 8.15, and 48.13 ± 4.56 mg QE/g extract, respectively, as shown in the Table 3. Comparatively, *T. cordifolia* exhibited the highest flavonoid content among the three extracts, while *M. charantia* had significantly lower flavonoid content as shown in bar diagram Figure (2b).

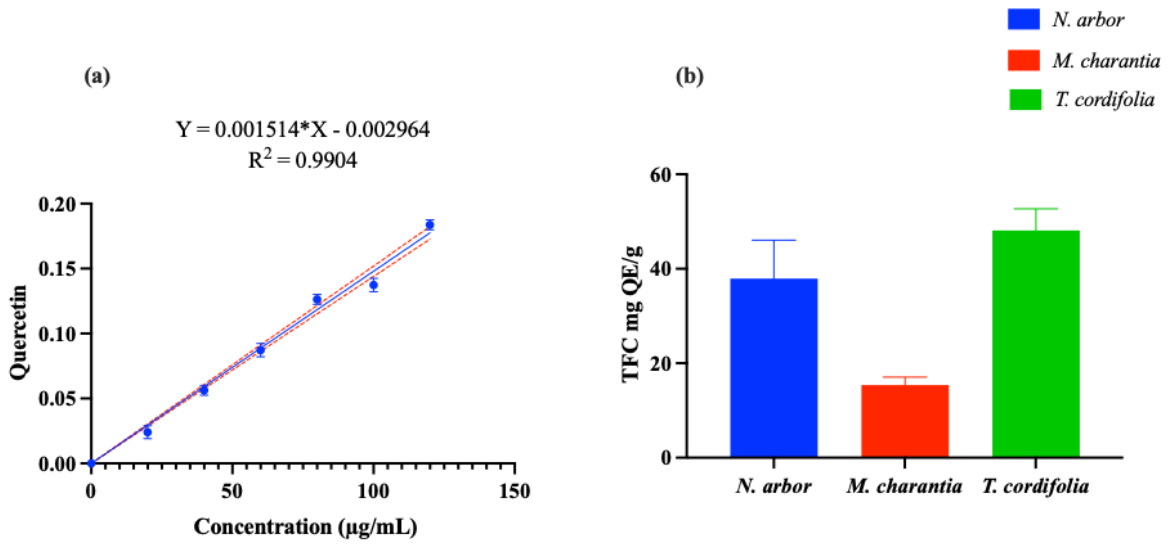

Figure 2. (a) Quercetin standard curve; (b) Bar-graph showing the total flavonoid content (mg QE / gram of dry extract)

This study suggests a similarity with many researcher investigations. A study conducted by Chokki *et al.* reported a higher value of 123.09 ± 9.63 µg QE/mg (Chokki *et al.*, 2020) in the methanolic extract of *M. charantia* relating to our results. In contrast, Khan *et al.* investigated a lower value of 21.97±0.98 mg QE/g of dry extract for the methanolic extract of *T. cordifolia* (Khan *et al.*, 2020) compared to our findings. Therefore, several factors influenced the obtained results, such as the structural complexity of flavonoids, choice of solvents (Nawaz *et al.*, 2020), analytical testing methods (Abubakar & Haque, 2020), selection of the plants, and the presence of interfering substances. Additionally, the total flavonoid content may be influenced by various environmental factors like temperature (Albert *et al.*, 2009), light (Pereira, 2006), and photoperiodic conditions (Taylor, 1965). Even genes also play a crucial role in flavonoid biosynthesis (Koes *et al.,* 2005).

Antioxidant Assay

The percentage inhibition was calculated by using Equation (4) and positive control was taken as Ascorbic acid. The comparative antioxidant activity of the selected plant extracts was illustrated by plotting the inhibition and concentration curve, which is clearly presented in Figure (3a) (See Table S4a). A fifty-percentage inhibition concentration (IC_{50}) was evaluated by using graph pad prism 8.0.2. The obtained result was depicted in the bar diagram Figure (3b). Consequently, IC_{50} values of methanolic extracts of *M. charantia*, *N. arbor*, *T. cordifolia,* and standard ascorbic acid were 69.34 ± 1.09, 48.16 ± 1.03, 45.07 ± 0.09, and 31.075 ± 1.04 µg/mL, which were shown in Table 4. When compared to the ascorbic acid, only the methanol extract of *T. cordifolia* (45.07 ± 0.09 µg/mL) showed a level of antioxidant potential that is nearly close to ascorbic acid (31.075 ± 1.04 µg/mL). Conversely, *M. charantia* and *N. arbor* exhibited lower levels of antioxidant activity. Among the plant extracts, *T. cordifolia* demonstrated the highest antioxidant activity, followed by *N. arbor* and *M. charantia*.

The findings of this study were related to previous research. Chokki *et al.* reported an IC_{50} value of 1.30 ± 0.12 mg/mL for the methanol solvent of *M. charantia* (Chokki *et al.*, 2020), while Khan *et al.* observed an IC_{50} value of 9.36 ± 1.75 mg/mL for the methanolic extract of *T. cordifolia* (Khan *et al.*, 2020). These values indicated a lower antioxidant potential compared to the results obtained in our study. Similarly, Numer and Sharma found IC_{50} values of 36.08 ± 3.024 µg/mL for *T. cordifolia* (Alam & Sharma, 2020), further supporting the relatively lower antioxidant activity. Interestingly, Lin *et al.* investigated a methanol extract containing four compounds in the stem and fruit of *M. charantia* and reported IC_{50} values of 119.1 ± 4.3, 204.5 ± 1.2, 159.7 ± 11.0, and 98.1 ± 2.4 at one molar concentration (Lin *et al.,* 2011). These values were also lower than our obtained results, indicating comparatively lesser antioxidant potential. Furthermore, Ghosh *et al.* recorded a free radical scavenging efficacy of 91.9 ± 0.3% at 2 mg/mL in the leaves of *N. arbor* (Ghosh *et al.*, 2015). However, these findings appeared to be less potent compared to our obtained results. It is important to note that the antioxidant activity of plants can be influenced by various factors, such as the DPPH test showing greater antioxidant activity with small molecules (Kırca & Arslan, 2008), as well as the nature of phenolic and flavonoid compounds and their ability to donate electrons and hydrogen (Pietta *et al.,* 1998).

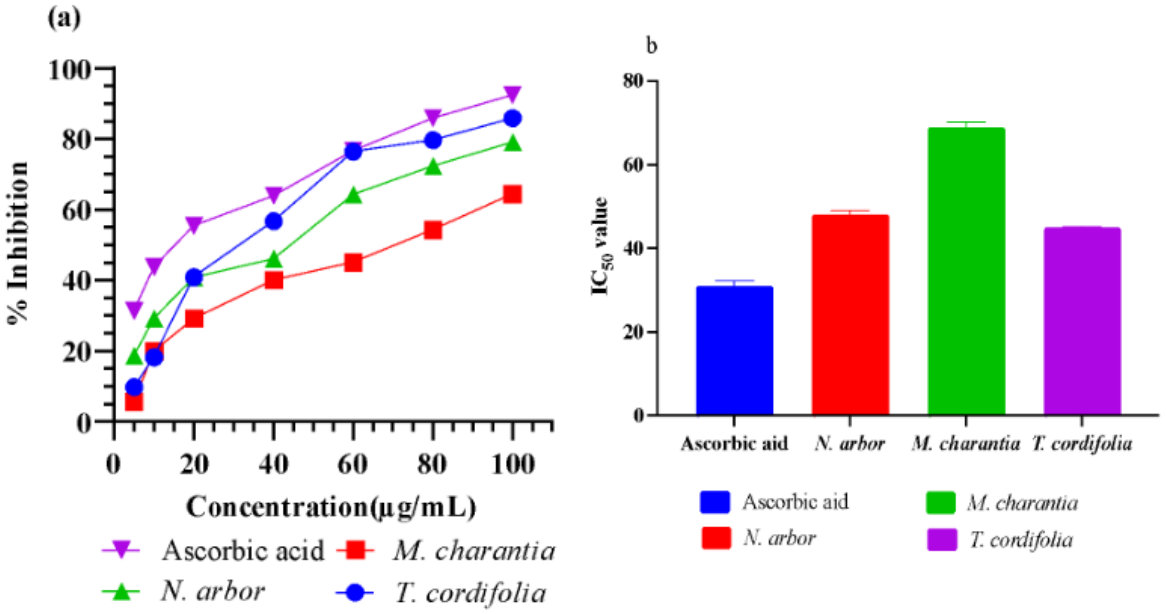

Figure 3. (a) A plot illustrating the percentage inhibition of methanolic extracts of *M. charantia, N. arbor, T. cordifolia*, and ascorbic acid against concentrations (µg/mL); (b) A bar graph showing the IC_{50} values for antioxidant inhibitory activity of ascorbic acid and methanolic plant extracts.

Brine Shrimp Bioassay

The toxicity analysis of methanolic extracts in *M. charantia*, *N. arbor*, and *T. cordifolia* was conducted at concentrations of 10, 100, and 1000 µg/mL. The biological activities of these plant extracts were evaluated based on their toxic effects on newly hatched brine shrimp nauplii. The LC_{50} values (µg/mL) were then determined for each concentration, and extracts with values below 1000 were considered to have pharmacological activity (See Table S4b). The result of this study shows LC_{50} values of *M. charantia* (316.22 ± 2.54 µg/mL), *N. arbor* (275.42 ± 1.78 µg/mL), and *T. cordifolia* (275.42 ± 1.63 µg/mL) respectively, which are presented in Table 4. Interestingly, all methanolic leaf extracts of *M. charantia*, *N. arbor*, and *T. cordifolia* exhibited LC_{50} values below 1000, which ensured their pharmacological activity. The highest mortality of brine shrimp larvae was observed at 1000 µg/mL, while the lowest number of deaths occurred at 10 µg/mL. These findings indicate a direct relationship between extract concentration and lethality.

Based on a literature survey, Shrestha and Lamichhane reported that the bioassay results reveal considerable cytotoxic activity in the methanolic extracts of *T. cordifolia*, showing a LC_{50} value of 232.64 ppm (Shrestha & Lamichhane, 2021). Uddin *et al.* found that the aqueous fraction of the crude methanol extract of *T. cordifolia* also displayed noteworthy toxic activity against brine shrimp nauplii, with a LC_{50} value of 2.246 mg/ml (Uddin *et al.*, 2011). Another study conducted by Amin *et al.* examined the lethality of methanol extract of *M. charantia* leaves on *Artemia salina*, *L. nauplii*, revealing LC_{50} values of 525.15 ppm, 107.71 ppm, and 41.67 ppm after 18, 24, and 30 hours of exposure (Amin *et al.*, 2017). Moniruzzaman *et al.* also reported a LC_{50} value of 122.11 ppm for *M. charantia* leaves (Moniruzzaman *et al.*, 2022). Regarding *N. arbor*, Haque *et al.* recorded a LC_{50} value of 0.50 ppm for the methanol extract (Haque *et al.*, 2020), while Fahim *et al.* suggested a LC_{50} value of 0.11 ppm for the methanolic leaf extract (Fahim & Karmakar, 2018). These findings highlight the significant pharmacological activity of the selected plants and bolster our own obtained results.

α-amylase Inhibition Assay

Initially, percentage inhibition was evaluated by using Equation 10 (see Table S4c). Then obtained results were compared with the standard acarbose positive control. These were represented prominently in Figure 4 (a to c), which follows the enzymatic curve. A fifty-percentage inhibitory concentration (IC_{50}) was measured from the data of our result by using a software Graphpad prism, and their IC_{50} values were found to be acarbose (85.40 ± 1.01 µg/mL), *T. cordifolia* (315.55 ± 1.03 µg/mL), *M. charantia* (507.72 ± 1.05 µg/mL), and *N. arbor* (530.26 ± 1.57 µg/mL) respectively as depicted in Table 4. These results were further compared in terms of the potency of the plant extracts and acarbose, as depicted in the bar diagram shown in Figure (4d). It is evident that among the three plants, *T. cordifolia* (315.55 ± 1.03 µg/mL) exhibited the highest inhibitory potential in inhibiting *α*-amylase, followed by *M. charantia* (507.72 ± 1.05 µg/mL), and *N. arbor* (530.26 ± 1.57 µg/mL) displayed the least inhibitory potential. However, when compared to the standard acarbose, all three extracts were found to be less potent.

Table 4. Biological activities of selected plants such as Antioxidant, Brine shrimp test and *α*-amylase inhibition

S.N	Name of plant	Antioxidant activity (IC_{50} µg/mL)	Brine shrimp lethal test (LC_{50} µg/mL)	*a*-amylase inhibition (IC_{50} µg/mL)
1	*M. charantia*	69.34 ± 1.09	316.22	507.72 ± 1.05
2	*N. arbor*	48.16 ± 1.03	275.42	530.26 ± 1.57
3	*T. cordifolia*	45.07 ± 0.09	275.42	315.55 ± 1.03
4	**Ascorbic acid**	**31.075** ± 1.04	**-**	**-**
5	Acarbose	-	-	85.40 ± 1.01

Note:- Overall comparative analysis of biological activity, where – sign indicates no test were done; IC_{50} and LC50 represents the fifty percentage concentration that extracts can inhibits or toxic to kill fifty percentage of larva of shrimp.

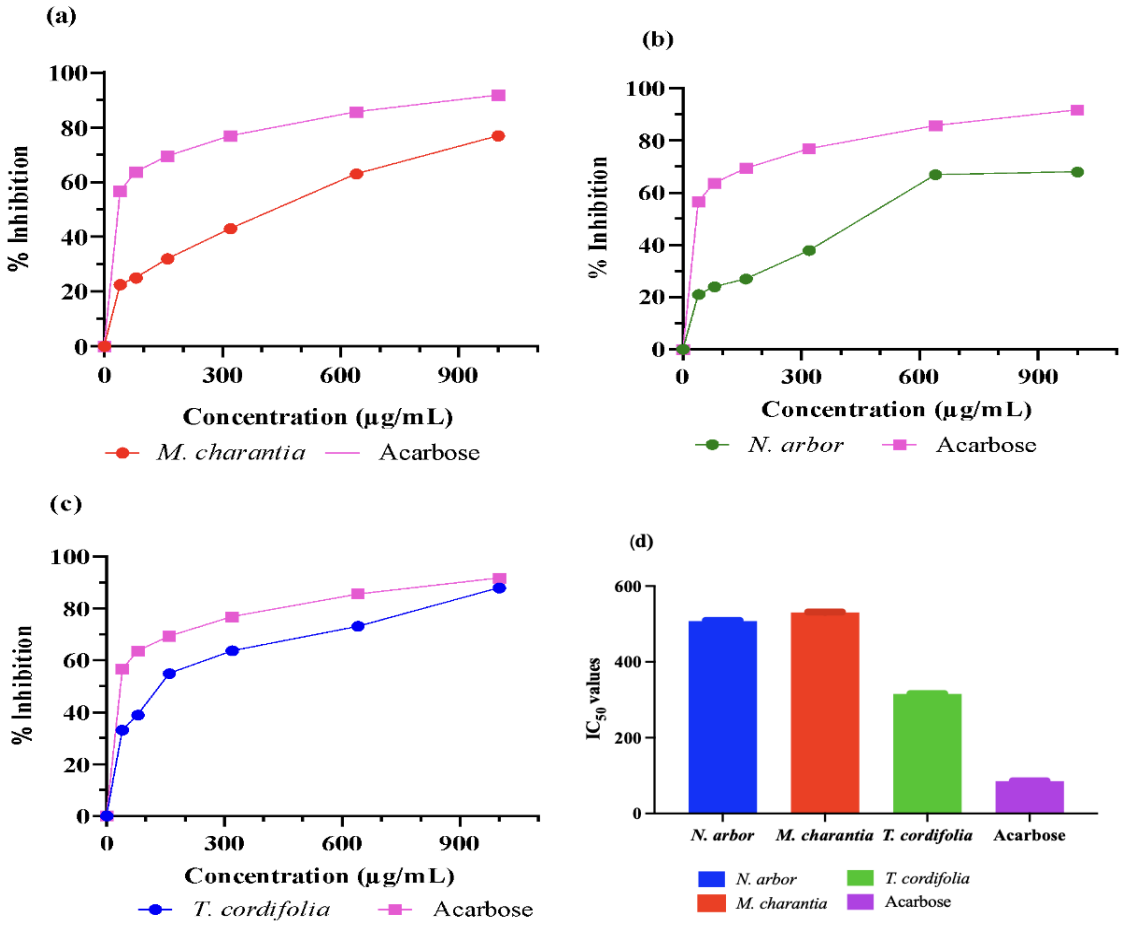

Figure 4. Displays a graph comparing different plant extracts with the standard acarbose: (a) A comparison between standard acarbose and *M. charantia*; (b) A comparison between standard acarbose and *N. arbor*; (c) A comparison between standard acarbose and *T. cordifolia*; (d) A bar graph.

Furthermore, the results of this assay are consistent with other investigations conducted on these plants. Sudha *et al.,* 2011 reported that the methanol extract of *T. cordifolia* did not exhibit any inhibition (Sudha *et al.*, 2011). However, our findings demonstrate its potency in inhibiting *a*-amylase. Patel and Mishra also suggested that the alkaloid derived from the methanolic extract of *T. cordifolia* reduced hyperglycemia at concentrations higher than 20 µg/mL (Patel & Mishra, 2011). Interestingly, Gaurav *et al.* study on the Ayurveda polyherbal tablet BGR-34, which contains *T. cordifolia*, revealed an inhibition of *a*-amylase with an IC_{50} value of 45.65 ± 1.855 µg/mL (Gaurav *et al.*, 2020). Shivanagoudra *et al.* (2019) recorded that a compound extracted from the methanol extract of *M. charantia* exhibited a 70.5% inhibition of *a*-amylase. Additionally, Nhiem *et al.* (2010) isolated fourteen cucurbitane type triterpene glycosides from the methanolic extract of bitter melon and found glucosidase inhibition of 21.71% and 18.63% at a concentration of 50 µM. Therefore, the comparative study suggests the potential ability of these

plants to inhibit *a*-amylase, which could be a valuable source for the development of new drugs.

FTIR and GC-MS analysis

Subsequent to column chromatography, the resulting yellow needle-shaped crystals were eluted using a solvent mixture of 13% ethyl acetate in hexane. The crystals were further purified through several hexane washings and subsequent recrystallization. Following the acquisition of the needle-shaped yellow crystalline substance on elution fractionT26, it underwent analysis via thin layer chromatography (TLC) utilizing a solvent mixture of 15% ethyl acetate in hexane. The compound displayed a singular spot on the TLC plate, with an Rf value of 0.58. FTIR spectroscopy was subsequently conducted on T26, and the resulting spectrum is illustrated in Figure S3a**.** FTIR is a technique employed to obtain an infrared spectrum of a given sample, allowing for the detection of various functional groups. The observed FTIR spectrum of T26 displayed a broad peak at 1000 cm^{-1}, potentially attributed to C-O stretching or bending. Additionally, a sharp peak at 2900 cm^{-1} suggested C-H stretching, while a multiplet around 3600 cm^{-1} indicated the presence of an O-H group.

The hexane fraction of methanol extract was also subjected to GC-MS analysis, which yielded a gas chromatogram displaying a total of 50 peaks. Table 5 lists some of the major constituents present in the chromatogram, while Figure 6 outlines the possible compounds detected. The resultant compounds exhibit functional groups such as –OH and C-O, which are identified in the IR spectra in this study (Figure S3a).

Table 5. Major chemical constituents detected by GC-MS

Peak	Area%	Retention Time	Name	Base m/z
1	6.867	14.84	Hexanoic acid, 6-amino-6-oxo-	45.10
2	15.399	23.48	2H-Pyran-2,4(3H)-dione dihydro- 6-(4-fluorophenyl)-3,3-dimethyl-5-spirocyclohexane- 3	194.20
3	21.442	15.04	Benzo[b]thiophene-2- carboxamide,3-chloro-N-(2-bromophenyl)-	109.00
4	22.108	21.80	9,12,15,18,21,24-Hexaoxa(2,16)[30]paracyclophane	45.10

Note: - shows a possible compound in the extracts of N. arbor fraction T26 along with the percentage area and retention time.

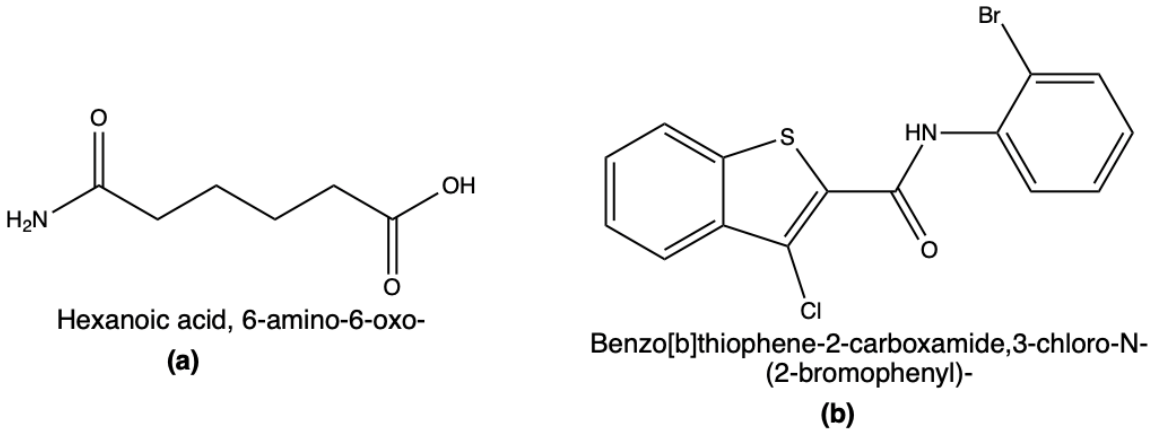

Figure 6. Major compounds in the hexane fraction of *N. arbor* detected by GC-MS

CONCLUSIONS

This study concludes that the methanolic extracts of plants including *M. charantia*, *N. arbor*, and *T. cordifolia*, collected from Lamahi Dang, Nepal, are rich in bioactive phytochemical constituents such as polyphenols, flavonoids, alkaloids, tannins, and saponins. The results of the biological activity assessments clearly indicate that among the three plants, *T. cordifolia* exhibits greater potential for antioxidant activity, *a*-amylase inhibition, and lower toxicity compared to the other extracts. Similarly, *N. arbor* demonstrates higher efficiency in toxicity tests and *a*-amylase inhibition activity compared to *M. charantia*. However, the antioxidant assay reveals that *M. charantia* exhibits higher antioxidant activity than *N. arbor*. Furthermore, in the case of *N. arbor*, possible compounds were isolated and characterized using FTIR and GC-MS analysis. The current study confirms that the selected plants in this research possess biological activity and are rich in phytochemicals. These plants have the potential to play a significant role in drug design research. Further studies are needed to perform chemical profiling and *in vivo* biological tests.

AUTHORS CONTRIBUTIONS

TS: Carried out the experiment; KC, BBK, and RP: wrote the manuscript with input from all authors; BS: conceived the original idea and supervised the project.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding author, upon reasonable request.

Supplementary Materials

Table S3a: Absorbance of standard Gallic acid at different concentrations, Table S3b: Absorbance of plants extract at different concentration for the determination of total phenolic content, Table S3c: Absorbance of standard Quercetin at different concentrations, Table S3d: Absorbance of plants extract at different concentration for the determination of total flavonoid content, Table S4a: Absorbance of different plants extract at different concentrations and their IC_{50} values, Table S4b: LC_{50} values for various plants extract. Table S4c: Percentage inhibition of plants extracts and acarbose against the *α*-amylase. Figure S3a: FTIR spectra of fraction T26 *N. arbor*. Figure S3b: Map of Nepal showing Area of Study, Dang district in red color.

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